Human Keratinocyte Locomotion: The Effect of Selected Cytokines

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Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) are two powerful mitogens for human keratinocytes that also have been shown to promote the healing of in vivo wounds. Transforming growth factor-beta (TGF-β) markedly inhibits human keratinocyte proliferation and growth and yet has been shown to promote wound healing.

Using a migration assay that evaluates pure cell locomotion independently from cell proliferation, we examined the influence of EGF, bFGF, and TGF-B on human keratinocyte locomotion. Although these agents had profound influences upon the growth potential of keratinocytes in parallel thymidine incorporation assays, they had no significant effect upon keratinocyte locomotion when cells were apposed to either tissue culture plastic or a collagen substratum. In contrast, we found that bovine pituitary extract (BPE), a poorly defined mitogen that is commonly used in keratinocyte cultures, could stimulate keratinocyte locomotion when the cells were apposed to a collagen substrate. These studies demonstrate that i) keratinocyte locomotion and proliferation operate by completely independent mechanisms, ii) the positive effects upon wound healing by EGF, bFGF, and TGF-β are not due to a direct promotion of keratinocyte locomotion, and iii) that one or more components of BPE are capable of directly promoting keratinocyte locomotion on collagen. *J Invest Dermatol 98:12–16, 1992*

Keratinocyte migration across the wound bed is an early and critical event in the wound-healing process [1]. Re-epithelialization of a cutaneous wound comprises both keratinocyte migration and cellular division [1]. Human keratinocytes are stimulated to migrate when they are in contact with fibronectin, dermal interstitial collagen, and basement membrane-specific collagen [2,3]. In contrast, keratinocytes in contact with laminin, a major basement membrane glycoprotein, are inhibited from migrating on tissue-culture plastic or on a collagen matrix [3]. Growth factors such as epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), transforming growth factor-beta (TGF-β), and extracts of bovine pituitary glands (BPE) are known to have profound influences upon the proliferative potential of human keratinocytes [4,5–7]. However, there is little information about how these growth factors influence pure keratinocyte locomotion.

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Abbreviations:

bFGF: basic fibroblast growth factor
BPE: bovine pituitary extract
EGF: epidermal growth factor
MI: migration index
TGF-β: transforming growth factor-beta

Many experimental systems, such as those in which skin explants are placed on matrices and examined for the size of the keratinocyte outgrowth in short-term culture, have been used to assess keratinocyte migration [8,9]. However, an explant assay is a mixture of cellular movement and growth. In addition, explants also contain other living cells that have the potential to influence keratinocytes by the secretion of soluble cytokines. In an explant system, the added putative growth factor could have an effect upon fibroblasts or endothelial cells that could secrete one or more cytokines that secondarily affect keratinocyte migration and proliferation. Explant assays cannot measure pure keratinocyte locomotion.

In order to assess pure keratinocyte locomotion, a modification of the Albrecht-Buehler assay [10] was established for keratinocytes in which cellular locomotion could be distinguished from proliferation [2]. Using this assay, it was found that matrices profoundly influence keratinocyte movement [2,3]. The purpose of the current experiments reported here was to determine if well-known, soluble mitogens (EGF, bFGF, BPE) and anti-mitogen (TGF-β) could directly influence human keratinocyte locomotion.

MATERIALS AND METHODS

Cell Culture Human keratinocytes from neonatal foreskin were initiated into culture by the method of Rheinwald and Green [11] and subcultured in "complete MCDB 153 medium" with supplements [6]. All of the supplements were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise specified. The MCDB 153 medium was supplemented with EGF (10 ng/ml), insulin (5 μg/ml), hydrocortisone (0.4 μg/ml), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), bovine pituitary extract [6] (150 μg/ml), and six amino acids (histidine, 2.4×10^{-4} M; isoleucine, 7.5×10^{-4} M; methionine, 9×10^{-5} M; phenylalanine, 9×10^{-5} M; tryptophan, 4.5×10^{-5} M; and tyrosine, 7.5×10^{-5} M), and containing 0.1 mM Ca^{++}. These cultures have been shown to consist of basal keratinocytes and to be fibroblast free [12]. For each

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Table I. ^{3}H-thymidine Incorporation of Keratinocytes Treated With Soluble Factors

Addition	^{3}H-Thymidine Incorporated
None	800 ± 40[a]
TGF-β, 1 ng/ml	150 ± 30
TGF-β, 5 ng/ml	100 ± 40
TGF-β, 10 ng/ml	90 ± 10
bFGF, 0.5 ng/ml	1550 ± 30
bFGF, 5 ng/ml	1650 ± 150
bFGF, 10 ng/ml	1700 ± 40
EGF, 10 ng/ml	2500 ± 100
BPE, 140 μg/ml	3060 ± 20

[a] cpm, average ± ½ range of duplicate determinations.

migration assay (vide infra), keratinocytes were used between passages 3 and 5.

Matrix Molecules and Soluble Factors Pepsinized human placental type IV collagen (lot #89 F-3870) was obtained from Sigma. TGF-β (lot #901016) isolated from human platelets was purchased from Calbiochem, La Jolla, CA, and stored in siliconized glass tube aliquots at −70°C. Recombinant bFGF was graciously supplied by Dr. John Fiddes, California Biotechnology Inc., Mountain View, CA, and stored at 4°C until use. EGF was isolated from mouse submaxillary glands and purified to homogeneity according to Savage and Cohen [13] or purchased from Sigma Chemical Company (St. Louis, MO) and stored at −20°C until use. Bovine pituitaries were purchased from Pel-Freez Biologicals (Rogers, AR), extracted [6], and stored at −20°C (protein concentration 7 mg/ml).

Migration Assay A modification of the phagokinetic track assay of Albrecht-Buehler [10] was established for human keratinocytes [2,3]. Pure cell locomotion quantitated by computer-assisted image analysis as previously described [3]. Briefly, coverslips were plated with particulate gold salts and placed in 35-mm petri dishes. Extracellular matrix molecule (0–15 μg/ml) was added in Hanks' buffered salt solution and incubated for 2 h at 37°C; 10,000 keratinocytes were plated in each dish in "complete" MCDB 153 or in "incomplete" MCDB 153, which lacks BPE and EGF. The soluble factors, to be tested, were simultaneously added with the cells. In some experiments, the cells were preincubated for 48 h with the soluble factors before the migration assay. Each situation was performed in triplicate. Cultures were incubated for 20 h, washed, and fixed in 3% formaldehyde in phosphate-buffered saline. To quantitate migration, five random, non-overlapping fields from each dish were photographed and the percentage of the total field taken up by migration tracks was calculated for each field using a Summagraphics MM 1201 digitizing tablet connected to an IBM-XT computer. The percentage of total field area taken up by tracks was termed the migration index (MI). Confirmation of an observed difference in migration as statistically significant required rejection of the null hypothesis of no difference between mean migration indices obtained from each triplicate set (with five random, non-overlapping fields quantitated in each dish) at the $p = 0.05$ level using the Student t test.

Thymidine Incorporation Assay To assess the proliferative potential of human keratinocyte cultures to which mitogens or anti-mitogens were added, a keratinocyte thymidine incorporation assay as described by Chiu and O'Keefe [14] was used. Briefly keratinocytes were plated in 24-well trays in 0.5 ml of "incomplete" MCDB 153 and incubated for 7 h at 37°C. Control wells then received an additional 0.5 ml of "incomplete" medium, whereas experimental wells received 0.5 ml of "incomplete" medium along with soluble factors at the concentrations indicated in Table I. The incubation was continued for another 16 h. Each situation was performed in triplicate. One microcurie of [methyl-^{3}H] thymidine (specific activity 10 Ci/mmol, ICN, Irvine, CA) was then added to each well, and the incubation was continued for another 6 h. At the end of the incubation period, [^{3}H] thymidine incorporated into DNA was extracted and counted.

Enzyme-Linked Immunosorbant Assay The assay used to measure bFGF concentration in the culture media was a modified "sandwich" ELISA. Briefly, BALB/c 3T3 cells were grown to confluence in 96-well microtiter plates and then fixed with a solution of 5% paraformaldehyde, 1.2% glutaraldehyde in phosphate-buffered saline. Aliquots (5 μl) of standards or samples to be assayed were added to duplicate wells of the fixed cells along with 45 μl/well of binding medium [Dulbecco's modified Eagle's medium (DMEM-21) containing 0.1% gelatine and 50 mM HEPES, pH 7.5], and incubated overnight at 4°C to allow bFGF in the samples to bind to the low-affinity binding sites of the surface of the cells. To control for non-bFGF-mediated signal in the assay, the samples were also incubated with the cells in duplicate in the presence of 10 μg/ml heparin, which blocks bFGF binding to the cells. After the overnight incubation, the samples were washed from the wells, and the cells were treated first with a biotinylated anti-bFGF mouse monoclonal antibody (#48.1, raised at California Biotechnology Inc. by Dr. J. M. Scardina), then with streptavidin-conjugated alkaline phosphatase, and finally with paranitrophenylphosphate (PNPP). The production of p-nitrophenol from the PNPP by alkaline phosphatase was measured over time at 410 nm in an ELISA plate reader. The non-bFGF-mediated signal was generated in the assay (average absorbance in the duplicate wells containing heparin) was subtracted from the total signal (average absorbance in the duplicate wells containing no heparin), and the concentration of bFGF corresponding to the resulting absorbance was read off of a standard curve generated on the same assay plate.

RESULTS

Human keratinocytes apposed to a collagen matrix demonstrate high levels of migration [3]. In order to assure that the keratinocytes in each assay were healthy and that the assay was working as expected, a "positive control set" in which keratinocytes were plated on collagen in "complete medium" was part of each experiment. In addition, each experiment contained a "negative control set" in which keratinocytes were plated on tissue culture plastic. As shown in Fig 1, keratinocytes cultured in "complete medium" *(black bars)* demonstrate markedly enhanced locomotion on collagen compared

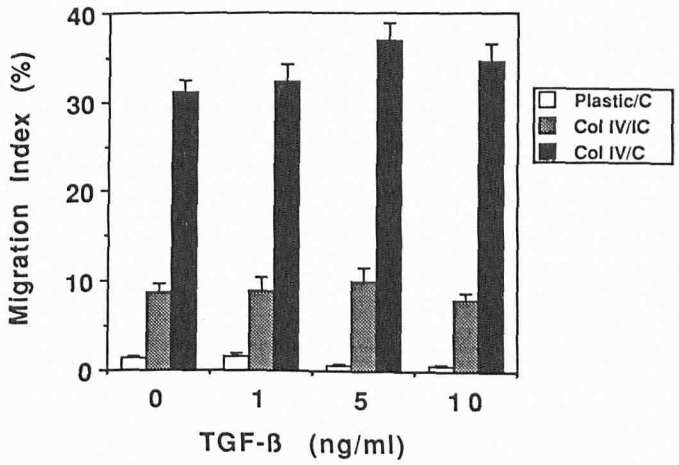

Figure 1. Migration indices of human keratinocytes apposed to type IV collagen and cultured in "complete" (C) or "incomplete" (IC) medium without or with the presence of TGF-B. *Error bars,* SEM of 15 or more fields. Student t test analysis of comparisons made between identical cultures with and without the presence of TGF-B produced p values greater than 0.01, indicating that the small differences observed in the presence of TGF-B were not statistically significant. In contrast, the p values were less than 0.005 when cells apposed to a collagen matrix were compared to those apposed to tissue culture plastic regardless of the medium used or the presence of TGF-B.

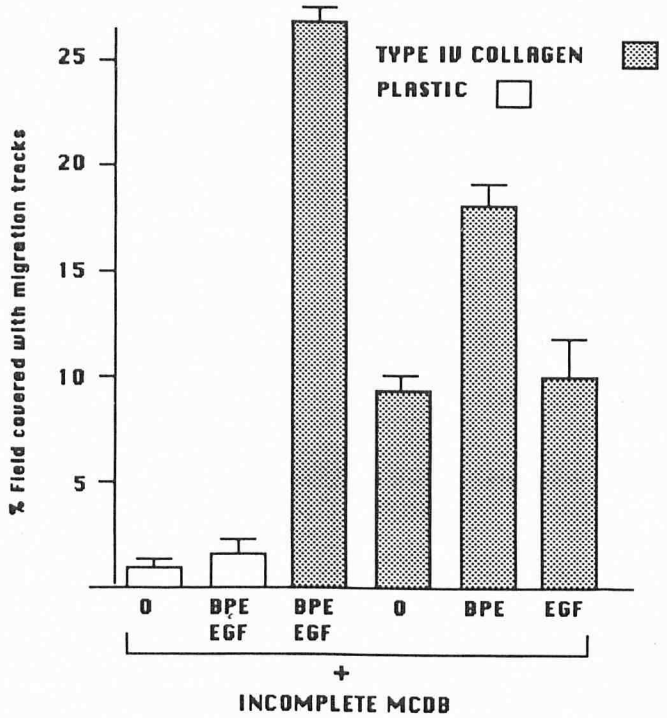

Figure 2. EGF and BPE effects on cell locomotion. Migration indices of human keratinocytes cultured on collagen IV and in presence or absence of EGF (10 ng/ml) and/or BPE (140 μg/ml). *Error bars,* SEM of fifteen or more fields. Student t test analysis comparing cells apposed to a collagen matrix in the presence of BPE and EGF (column 3) with those on plastic (columns 1 and 2) gave p values less than 0.005. A comparison of cells migrating on collagen in "incomplete" medium without BPE (columns 4 and 6) or with BPE (column 5) generated a p value of 0.003. Cells apposed to type IV collagen in "incomplete" medium without BPE (column 4) compared with those without BPE but with EGF resulted in a p value greater than 0.01.

to cells juxtapposed to tissue culture plastic *(white bars)*. The withdrawal of EGF and BPE from the "complete" medium (*grey bars,* "incomplete" medium) induced a 70% reduction of cell motility on collagen.

As also demonstrated in Fig 1, the addition of various concentrations of TGF-β, a potent anti-mitogen, had no significant impact upon the keratinocyte's ability to migrate on collagen in either "complete" or "incomplete" medium. In addition, TGF-β did not appear to significantly promote the locomotion of keratinocytes when they were apposed to either tissue-culture plastic or collagen.

In order to be sure that TGF-β was biologically active, keratinocyte thymidine incorporation assays were performed in parallel with the locomotion assays. As shown in Table I, the addition of TGF-β to the identical cells used in the locomotion assay inhibited the incorporation of thymidine by 90%.

EGF and BPE are two mitogens that are commonly added to human keratinocyte cultures in order to promote cell growth [6,7]. These mitogens are constitutive components of "complete" MCDB 153 with supplements, the medium used in our routine cultures and migration assay. In order to assess their role in keratinocyte migration, we excluded EGF and BPE from the routine medium and designated it as "incomplete" medium. Keratinocyte migration on a collagen substrate was significantly decreased when EGF and BPE were excluded from the medium (Figs 1 and 2). The baseline migration index of keratinocytes apposed to type IV collagen fell from 28% ($\pm$1) in "complete" medium to 9.9% ($\pm$0.7) when EGF and BPE were eliminated from the medium. The addition of EGF alone to "incomplete" medium did not significantly restore migration towards the level seen when complete medium was used (Fig 2). In contrast to EGF, the addition of BPE alone was capable of partially restoring keratinocyte migration as demonstrated by an approximately twofold increase in the MI.

In order to decrease the influence of the matrix and perhaps reveal or enhance an effect of one or more of the soluble factors, parallel experiments were performed in which the keratinocytes were cultured under identical conditions but on a suboptimal collagen matrix. When the cells were apposed to a suboptimal matrix of 4 μg of type IV collagen, the migration index in "incomplete" medium was 6.5% ($\pm$0.8, compared to 9.9% when the cells were apposed to 15 μg of type IV collagen, see above). The addition of BPE to cells apposed to 4 μg of type IV collagen increased the migration index to from 6.5% to 13.9% ($\pm$0.5), a restoration of the MI to 58.4% of that observed when the cells were apposed to the same matrix in "complete" medium. Therefore, the addition of BPE to "incomplete" medium enhanced the migration of keratinocytes on either 4 or 15 μg of type IV collagen by approximately the same proportions relative to control cultures in "complete" medium. The addition of BPE to "incomplete" medium did not produce a noticeable enhancement of migration when the keratinocytes were apposed to tissue-culture plastic or albumin. However, this is probably because of the very low levels of migration when the cells are not apposed to components of the extracellular matrix (Fig 2).

BPE is known to contain significant amounts of bFGF [15]. Using a modified "sandwich" ELISA, we found that the bFGF concentration in "complete" medium ranged from 0.65 – 1.07 ng/ml. In "incomplete" medium, the bFGF concentration was below the level of detection by the assay, which is sensitive down to 50 pgs/ml. As shown by O'Keefe et al [5], bFGF is a potent mitogen for human keratinocytes. In order to assess whether or not the enhancement of keratinocyte migration on collagen IV by BPE was due to bFGF, experiments were designed in which locomotion assays were performed in "complete" and "incomplete" medium with or without the addition of various amounts of bFGF, which were chosen to be above and below the bFGF concentration in the "complete" medium. To be certain that the bFGF additions were sufficient to invoke a biologic effect, parallel thymidine incorporation assays were performed with and without the presence of bFGF. As shown in Fig 3, the addition of bFGF to "complete" medium or "incomplete" medium did not enhance keratinocyte migration on either a collagen matrix or on tissue culture plastic. However, as expected [5], the parallel thymidine incorporation assay (Table I) demonstrated that the bFGF addition increased thymidine incorporation twofold above control.

It has been shown previously that keratinocytes apposed to a collagen or fibronectin matrix are stimulated to migrate [2,3]. Maximal stimulation of migration occurs when between 15 and 30 μg per ml of collagen are adsorbed to the bottom of 35-mm petri dishes [3]. We considered the possibility that this matrix stimulation of migration may be so powerful that it might "override" any effect of a less potent soluble factor. If this were to occur, we would perhaps miss the more subtle effects of the soluble factors. In order to examine this possibility, we performed locomotion assays on submaximal amounts of collagen substrate with and without the presence of TGF-β and bFGF. After establishing a dose-dependency curve for keratinocyte locomotion on decreasing amounts of collagen substrate adsorbed to the dishes, we chose a concentration of 4 μg/ml of type IV collagen. This amount of collagen gave a 60% reduction in the MI compared to our routine assay in which 15 μg/ml is added, but still allowed detectable migration and linear track formation (data not shown). On submaximal amounts of type IV collagen, keratinocyte migration was not enhanced by the addition of TGF-β or bFGF over concentrations ranging from 1 to 10 ng/ml (not shown).

In order to be sure that the exposure time of the keratinocytes to TGF-β and bFGF was not too short, cells were cultured in the presence of TGF-β (10 ng/ml) or bFGF (10 ng/ml) for 48 h prior to and during the migration assay. As shown in Fig 4, neither TGF-β or bFGF significantly enhanced keratinocyte migration on type IV collagen or plastic over controls.

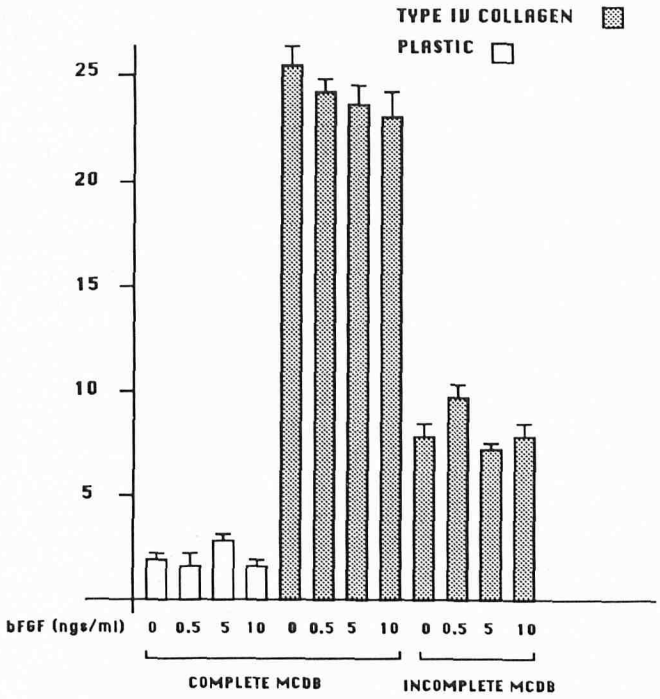

Figure 3. Migration indices of human keratinocytes in the presence of bFGF. Cells were cultured in the absence or presence of type IV collagen (15 μg/ml), in the presence of various amounts of bFGF and in "complete" or "incomplete" medium. *Error bars,* SEM of fifteen or more fields. Student t test analysis of comparisons made between identical cultures (i.e., same medium and matrix) with and without the presence of bFGF produced p values greater than 0.01. Therefore the small differences observed between control and experimental situations were not statistically significant.

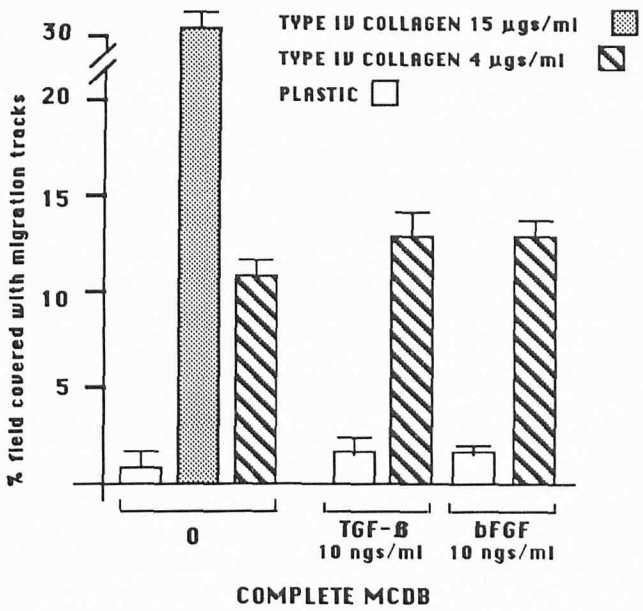

Figure 4. Migration indices of human keratinocytes. Cells were cultured in the presence of TGF-β or bFGF 48 h prior to and during the migration assay and plated on submaximal amounts of type IV collagen or on plastic. Non-pretreated cells were used in parallel. *Error bars,* SEM of 15 or more fields. Although a small enhancement of keratinocyte migration on suboptimal amounts of type IV collagen *(striped bars)* are noted when TGF-B and bFGF are present, Student t test analysis of these comparisons produced p values greater than 0.01.

DISCUSSION

EGF, bFGF, and TGF-β have all been shown to enhance wound healing in animals or human beings [16–20]. However, the mechanisms by which these factors exert this effect are unknown. One possible mechanism is that these factors could directly promote keratinocyte migration, an early and critical event in wound healing. Our keratinocyte migration assay is designed to specifically examine pure cell locomotion that is not confounded by cell division. Over all, the data reported here suggest that the improvements observed in wound healing by the presence of EGF, bFGF, and TGF-β are not due to enhanced keratinocyte migration. This does not mean that these soluble factors are not helpful for in vivo wounds because wound healing is a complex process that includes a number of other biologic parameters besides keratinocyte migration, such as coagulation, fibrin clot formation, inflammation, angiogenesis, contraction, and connective tissue synthesis and remodeling [1]. Although we could not demonstrate a significant enhancement of keratinocyte locomotion by any of these growth factors, it may be that they promote wound healing by some other mechanism or by their influence upon another cell type such as fibroblasts, which then secondarily influence keratinocyte locomotion. We are confident that all of the soluble factors used in this study were active because their expected biologic effects upon keratinocyte growth potential were readily demonstrated in parallel, independent thymidine incorporation assays.

TGF-β did show a minimal enhancement of keratinocyte migration when keratinocytes were apposed to collagen or plastic (Figs 1 and 4), but we do not feel that this is significant when compared with the effects that can be readily achieved with matrices alone on keratinocyte locomotion [2,3], and the Student t test analysis never reached significance. Nickoloff et al [21], using two other types of keratinocyte locomotion assays, an agarose drop explant technique, and a micropore filter assay, was able to demonstrate enhanced locomotion of keratinocytes on tissue-culture plastic in the presence of TGF-β. The disparity between this study and the results shown here is not clear.

The experiments here suggest that a component of BPE can support enhanced keratinocyte migration on collagen (Fig 2). Although BPE is rich in bFGF, the component that supports keratinocyte migration appears not to be bFGF, because the addition of this factor to "incomplete" medium could not substitute for BPE. The nature of the component within BPE that partially restores keratinocyte migration is not known. Whatever the component is, it appears that BPE operates synergistically with EGF because EGF plus BPE (i.e., "complete" medium) supported keratinocyte locomotion on collagen more than the same medium without EGF (Fig 2). Moreover, although BPE enhanced locomotion when the cells were apposed to suboptimal amounts of type IV collagen (4 μg), the fact that locomotion was greater in the presence of BPE when the cells migrated on 15 μg of type IV collagen suggests synergy between the matrix and the growth factor. These findings suggest that there is a degree of complexity with regard to soluble factors and matrix that may support keratinocyte locomotion in concert. In addition, there may be soluble co-factors that work in concert to maximally stimulate keratinocytes to migrate.

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